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Endotoxin-Neutralizing Antimicrobial Proteins of the Human Placenta[1]

Hun Sik Kim,* Ju Hyun Cho,* Hyoung Woo Park,† Ho Yoon,‡ Mi Sun Kim,§ and Sun Chang Kim[2]*

Microbial colonization and infection of placental tissues often lead to adverse pregnancy outcomes such as preterm birth, a leading cause of neonatal morbidity and mortality. The fetal membranes of the placenta, a physical and active barrier to microbial invasion, encapsulate the fetus and secure its intrauterine environment. To examine the innate defense system of the human placenta, antimicrobial peptides were isolated from the fetal membranes of human placenta and characterized biochemically. Two salt-resistant antimicrobial host proteins were purified to homogeneity using heparin-affinity and reversed-phase HPLC. Characterization of these proteins revealed that they are identical to histones H2A and H2B. Histones H2A and H2B showed dose-dependent inhibition of the endotoxin activity of LPS and inhibited this activity by binding to and therefore blocking both the core and lipid A moieties of LPS. Consistent with a role for histones in the establishment of placental innate defense, histones H2A and H2B were highly expressed in the cytoplasm of syncytiotrophoblasts and amnion cells, where the histone proteins were localized mainly to the epithelial surface. Furthermore, culturing of amnion-derived WISH cells led to the constitutive release of histone H2B, and histones H2A and H2B contribute to bactericidal activity of amniotic fluid. Our studies suggest that histones H2A and H2B may endow the epithelium of the placenta with an antimicrobial and endotoxin-neutralizing barrier against microorganisms that invade this immune-privileged site. ***The Journal of Immunology*****, 2002, 168: 2356–2364.**

Pregnancy is an immunological balancing act in which the mother's immune system has to remain tolerant of allogeneic fetal tissues and yet maintain normal immune competence for defense against invading microorganisms (1, 2). Many studies indicate that suppression of maternal immunity provides an immune-privileged microenvironment for the fetus. Cytokines (3), the nonclassical MHC molecule HLA-G (4), Fas (5), TNF-related ligands (6), suppression of the complement system (7), and possibly catabolizing enzymes for T cell growth-promoting nutrients (8) expressed at the decidual-placental interface all have been implicated in the modulation of maternal immune competence. However, this host immune suppression for allograft survival (9) does leave the placenta and fetus more susceptible to microbial infection.

Preterm birth remains the major cause of neonatal morbidity and mortality. Although the etiology of preterm labor and delivery is unknown, there is overwhelming epidemiological, pathological, and immunological evidence to implicate the infection of the uteroplacental unit as a causative factor in a substantial number of preterm births (10–12). Infection-related prematurity is most likely caused by an infection that ascends from the lower genital tract to the upper tract, with the end stage being a positive amniotic fluid culture (13, 14). Fetal exposure to high concentrations of microorganisms leads to colonization of the fetus lung airways and subsequent pneumonia, sepsis, and meningitis (15, 16). Even a trivial fetal infection can trigger an exaggerated inflammatory response, inducing cytokine cascades and resulting in the eventual demise of the neonate (17). Despite the large number of infectious insults during pregnancy, the outcome is most often a normal newborn. This is undoubtedly due to the presence of highly effective fetal defense mechanisms that serve to limit the bacterial infection of placental tissues. The placenta is a highly selective mechanical and immunological barrier against the dissemination of infectious agents by hematogenous routes or by the ascending route from the vagina (18). These defenses serve to limit both the degree and frequency of bacterial colonization within the placenta. Therefore, the placenta may contain effective protection mechanisms that, upon their failure, permit the establishment of a bacterial infection in fetal tissues.

During the last several decades, it became clear that antimicrobial proteins serve a key protective role in the host defense of widely divergent animal species. Such proteins are active components of the innate defense system, acting as effector molecules with the capacity to kill a broad spectrum of microorganisms (19–21). In contrast to highly specific adaptive immunity, which is not triggered rapidly enough to protect against exposure to pathogens, the innate immune system provides a rapid and nonspecific response, and thereby contributes to the first line of defense against infection. The recent discovery of antimicrobial proteins within the human respiratory epithelium (22, 23), the gastrointestinal epithelium (24, 25), Paneth cells of the small intestine (26, 27), and the urogenital epithelium (28) has prompted us to examine placental tissue and associated membranes for similar agents. From the fetal membrane of human placenta, we have isolated two salt-resistant proteins with antimicrobial and endotoxin-neutralizing activities. These proteins were characterized and shown to be histones H2A

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and H2B. H2A and H2B were expressed in the cytoplasm of syncytiotrophoblasts and amnion cells, where they were associated mainly with the surface epithelium. In addition, amnion epithelial cells were shown to release histone H2B constitutively. Our findings suggest that histones H2A and H2B may provide protection against penetration by microorganisms into the immune-privileged human placental tissues.

Materials and Methods

Materials

Polymixin B (PMB),[3] PMB nonapeptide (PMBN), *Escherichia coli* 0111:B4 LPS, *E. coli* EH100 (Ra mutant) LPS, *E. coli* J5 (Rc mutant) LPS, *E. coli* F583 (Rd_2 mutant) LPS, and *E. coli* lipid A were purchased from Sigma-Aldrich (St. Louis, MO). DMEM, FBS, penicillin, and streptomycin were obtained from Life Technologies (Gaithersburg, MD). Calf thymus histones H2A and H2B were from Roche (Mannheim, Germany). Anti-histone H2A (BWA3) and anti-histone H2B (LG2-2) mAbs were kindly provided by Dr. M. Monestier (Temple University School of Medicine, Philadelphia, PA).

Tissues

Samples of human second trimester ($n = 5$) and term placentas ($n = 3$) were obtained from elective pregnancy terminations and normal cesarean section deliveries, respectively, in accordance with a protocol approved by the Human Subjects Committee of the University of Yonsei Medical Center (Seoul, Korea). The tissues were manually dissected and processed immediately.

Amniotic fluid collection

Informed consents were obtained from all patients from whom amniotic fluid samples were collected. Amniotic fluid samples at term pregnancy were obtained from 20 patients who were not in labor and were undergoing amniocentesis for fetal lung maturity before scheduled elective repeated cesarean deliveries. All amniotic fluid samples selected for analysis were free of contamination from blood components. Amniotic fluid samples were centrifuged, and the supernatant was stored at −80°C until analyzed.

Purification of antimicrobial proteins

Antimicrobial proteins were purified from term human placenta by a previously described method (29) with slight modifications. The placenta (tissue weight, 100 g) was homogenized using a Waring blender (Waring, New Hartford, CT) in 500 ml of acidic medium containing 1% (v/v) trifluoroacetic acid (TFA), 1 M of HCl, 5% (v/v) formic acid, 1% (w/v) NaCl, and pepstatin A at 10 μg/ml. The homogenate was centrifuged at 20,000 × *g* for 30 min in a Himac SCR20BR (Hitachi, Tokyo, Japan), and the supernatant was collected. The material in the supernatant was then subjected to reversed-phase concentration using a Sep-Pak C18 cartridge (Millipore, Milford, MA), which was activated with 80% acetonitrile containing 0.1% TFA and flushed with 0.1% TFA to remove the excess acetonitrile. After being loaded with the supernatant, the cartridge was washed with 20 ml of 0.1% TFA, and the material trapped in the Sep-Pak C18 cartridge was eluted with 6 ml of 80% acetonitrile containing 0.1% TFA. The eluate was then lyophilized and subsequently resuspended in 10 ml of 0.01 M of Tris-HCl (pH 7.5) containing 0.01 M of NaCl. The resuspended eluate was loaded onto a 1 × 10-cm heparin-Sepharose column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 0.01 M of Tris-HCl (pH 7.5) containing 0.01 M of NaCl. The bound material was eluted by a stepwise gradient of 0.5, 1, and 3 M of NaCl, and concentrated using a Sep-Pak C18 cartridge. Each eluted sample (5 μl) was then assayed for antimicrobial activity against *E. coli*. The active eluate was subjected to chromatography on a C4 reversed-phase HPLC column (5 μm, 4.6 × 250 mm, Vydac; Millipore). The proteins loaded onto the C4 column were eluted with a 0–100% gradient of buffer A (0.13% heptafluorobutyric acid (HFBA) in water) and buffer B (0.13% HFBA in acetonitrile) at a flow rate of 1 ml/min over 2 h. Each fraction was dried under vacuum and resuspended in water. A sample of the resuspended proteins (5 μl) was assayed for antimicrobial activity. Final purification of two active fractions was achieved by reversed-phase HPLC on a C4 column with a slower gradient using 0.1% TFA as an ion-pairing agent. The purity of the isolated proteins was assessed by reversed-phase HPLC and tricine SDS-PAGE. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as a standard.

Structural analyses

The m.w. of the antimicrobial proteins was determined by matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS; Kratos Kompact MALDI, Manchester, U.K.). Lyophilized protein (~1 nmol) was dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a platinum probe. After removing the solvent in warm air, the protein that had been adsorbed to the platinum probe was applied to a vacuum chamber and analyzed. Proteins were hydrolyzed by incubation in evacuated tubes with 6 M of HCl containing 0.1% phenol at 110°C for 24 h. Total composition of the proteins was determined using a Pico-tag analysis system on a Beckman 121 MB amino acid analyzer (Fullerton, CA). The N-terminal sequence analysis of the proteins was performed by automatic Edman degradation on a gas-phase sequencer (Applied Biosystems, Foster City, CA).

Antimicrobial activity assay

The minimal inhibitory concentration (MIC) of the antimicrobial proteins was determined with a modified broth dilution assay (30). The test strain *E. coli* ML35 was inoculated into culture medium (3% trypticase soy broth) and cultured overnight at 37°C. An aliquot of this culture (50 μl) was transferred to 50 ml of fresh medium and incubated for an additional 3 h at 37°C so that the cells used in the assay are in mid-logarithmic phase. Bacteria were harvested by centrifugation, washed with 10 mM of sodium phosphate buffer (pH 7.4), and resuspended in the same buffer. A 2-fold dilution series of the purified proteins in 10 mM of sodium phosphate buffer was prepared, and serial dilutions (50 μl) were added to 50 μl of 5×10^4 CFU in each well of a static 96-well polypropylene microtiter plate (Costar, Corning, NY). Where indicated, fixed concentrations of NaCl were added to each well of the microtiter plate (Table I). After incubation for 3 h at 37°C, fresh medium was added to the mixture and incubated at 37°C for 16 h. The inhibition of growth was determined by measuring the absorbance at 620 nm with a model 550 microplate reader (Bio-Rad, Richmond, CA). The lowest concentration of peptide that completely inhibited bacterial growth was defined as the MIC. The MICs were obtained in triplicates on three independent measurements.

Measurement of LPS binding and neutralization

Assay for binding to immobilized LPS. The wells of a microtiter plate (PolySorp; Nunc, Roskilde, Denmark) were coated with LPS by incubating 100 μl/well of 30 μg/ml *E. coli* 0111:B4 LPS in 0.2 M of sodium carbonate-bicarbonate buffer (pH 9.4) for 1 h at 37°C. The LPS solution was removed, and the plates were blocked by incubation for 1 h at room temperature with TBS (pH 7.2) containing 3% (w/v) BSA. The blocked wells were then washed three times with 0.05% Tween 20 in TBS (TTBS). Histone H2B in TTBS (1 μg/ml) was added to each well in a total volume of 100 μl/well. To study the specificity of binding of histone H2B to LPS, various types of LPS (final concentration of 0.5–50 μg/ml) were added to each well of the microtiter plate immediately after the addition of histone H2B. Binding of histone H2B was allowed for 1 h at room temperature, and the microtiter plate was washed three times with TTBS. Anti-histone H2B mAb (1 μg/ml), 100 μl/well, in TTBS, was added to the plate, which was then incubated for 1 h at room temperature. The plate was then washed with TTBS as above and incubated for 1 h at room temperature with 100 μl/well peroxidase-conjugated goat anti-mouse IgG (1/5000 dilution) in

Table I. *Effect of NaCl on MICs of antimicrobial proteins against* E. coli

Additive	Conc[b] (mM)	MIC (μg/ml)[a] Histone H2A	Histone H2B	Magainin II
None		10	10	20
NaCl	100	10	10	20
	150	10	10	50
	200	15	15	100
	300	20	20	>200

[a] These are the average values of three experiments.
[b] Conc, concentration.

[3] Abbreviations used in this paper: PMB, polymyxin B; DAPI, 4′,6′-diamidino-2-phenylindole; HFBA, heptafluorobutyric acid; LAL, *Limulus* amebocyte lysate; MALDI-MS, matrix-associated laser desorption ionization mass spectroscopy; MIC, minimal inhibitory concentration; PMBN, PMB nonapeptide; TFA, trifluoroacetic acid.

TTBS. Immunoreactivity was visualized by adding 200 μl of 3,3′,5′,5-tetramethylbenzidine (Sigma-Aldrich) substrate solution. The reaction was stopped after 30 min by adding 100 μl of 0.18 M of sulfuric acid, and the absorbance at 450 nm was determined using a model 550 microplate reader (Bio-Rad).

Limulus *amebocyte lysate (LAL) assay.* The in vitro neutralization of LPS activity by histone proteins was assessed by the LAL chromogenic assay (QCL-1000; BioWhittaker, Walkersville, MD), according to the manufacturer's protocol.

TNF-α production by RAW264.7 cells. The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% heat-inactivated FBS. The cells were plated at 10^6 cells/ml/well in a 24-well plate (Nunc), incubated overnight, then stimulated with LPS (*E. coli* 0111:B4) at a final concentration of 10 ng/ml for 6 h. Immediately after LPS addition, histone proteins were added to a final concentration of 0.5–20 μg/ml. The culture supernatants were assayed for TNF-α using an ELISA (Roche). The viability of RAW264.7 cells was assessed by the viable cell proliferation assay (CellTiter 96 Aqueous One solution; Promega, Madison, WI), according to the manufacturer's protocol.

Immunohistochemistry

Fresh human placenta was placed in 10% formaldehyde dissolved in PBS, incubated overnight at 4°C, and embedded in paraffin. Serial cross sections (4 μm) were deparaffinized, treated with 0.3% hydrogen peroxide in methanol for 30 min, and washed extensively, first with water and then with PBS. Immunostaining was performed using a biotin-avidin-peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Briefly, the sections were incubated with 1% normal blocking serum for 20 min at room temperature and then incubated for 1 h with either anti-histone H2A (50 μg/ml) mAb or anti-histone H2B (10 μg/ml) mAb. Sections were developed with chromogen diaminobenzidine for 10 min and then counterstained with Mayer's hematoxylin and mounted. Control incubations were performed using mouse isotype-matched IgG as a primary Ab.

Immunocytochemistry

Fresh WISH cells were seeded into Lab-Tek tissue-culture chamber slides (Nunc) and grown for 24 h. Cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 or not permeabilized for immunolabeling of histones H2A and H2B at the surface of amnion WISH cells. After further washing, immunostaining was performed using a biotin-streptavidin-FluorGreen method (InnoGenex IHC Immunofluorescence kit; InnoGenex, San Ramon, CA). The cells were then blocked with a blocking reagent for 5 min at room temperature and incubated for 1 h with either anti-histone H2A (50 μg/ml) mAb or anti-histone H2B (10 μg/ml) mAb, biotinylated secondary Ab, and streptavidin-FluorGreen conjugate in sequence. The cells were rinsed, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), mounted, and viewed with confocal laser-scanning microscopy (LSM410; Zeiss, Oberkochen, Germany).

Amnion cell culture

The human amnion-derived cell line WISH was obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell cultures were transferred with 1 mM EDTA/0.05% trypsin (Life Technologies) upon reaching confluence and plated out at 1×10^5 cells/ml/well in a 24-well culture plate in the same media. To study the production of histone H2B in culture in response to LPS treatment, media samples were withdrawn and replaced with fresh media 24 h after plating. *E. coli* 0111:B4 LPS was added to the fresh media, and the cells were incubated for various indicated times. The conditioned media were then removed and centrifuged at $500 \times g$ for 10 min. The supernatants were recovered and stored at −20°C for immunoassay.

Measurement of histone H2B

The levels of histone H2B in the cultured WISH cell supernatant and amniotic fluid samples were determined with a sandwich ELISA. Microtiter plates (MaxiSorp; Nunc) were coated with 100 μl/well of 30 μg/ml DNA in 0.2 M of sodium carbonate-bicarbonate buffer (pH 9.4) by incubation for 1 h at 37°C. Plates were blocked for 1 h at room temperature with TBS (pH 7.2) containing 3% (w/v) BSA, and then washed three times with TTBS. Culture supernatants (100 μl) or serial dilutions of amniotic fluid samples (100 μl) were added to each well, and the reaction mixture was incubated for 1 h at room temperature. Subsequent incubations included anti-histone H2B mAb, followed by peroxidase-coupled goat anti-mouse IgG, each for 1 h at room temperature. The 3,3′,5′,5-tetramethylbenzidine substrate solution (200 μl) was then added to each well. The reaction was stopped after 20 min by adding 100 μl of 0.18 M of sulfuric acid, and the absorbance at 450 nm was determined using an ELISA reader. A standard curve was generated by adding known amounts of histone H2B to the assay.

Results

Purification of salt-resistant antimicrobial proteins from human placenta

Term placenta was homogenized in an acidic medium and fractionated on a heparin-Sepharose column. Antimicrobial activity against *E. coli* of the eluates from the heparin-Sepharose column was examined in the presence of 150 mM of NaCl. The active eluate was then subjected to reversed-phase HPLC, and two fractions were found to be responsible for the major salt-resistant antimicrobial activity (Fig. 1*A*). These fractions were further purified by a second reversed-phase HPLC separation. Each of the fractions contained a single protein band, as determined by tricine SDS-PAGE (Fig. 1*B*). The first protein eluted from the reversed-phase HPLC column was designated as peak 1 (m.w. of ~13,776.2 by MALDI-MS), and the second one was designated as peak 2 (m.w. of ~13,998.5 by MALDI-MS). The two antimicrobial protein preparations, which were at >95% purity as determined by reversed-phase HPLC, tricine SDS-PAGE, and MALDI-MS, were subjected to N-terminal sequence analysis (Fig. 1*C*). Peak 1 was sequenced up to 20-aa residues, and the sequence was PEPAKSAPAPKKGSKKAVTK. This sequence was found to be identical to the N-terminal sequence of human histone H2B (31). N-terminal sequencing of peak 2 gave us a very low yield, suggesting that it was N-terminally blocked. Thus, after an N-terminal deblocking step (32), peak 2 was sequenced up to 19 residues (SGRGKQG GKARAKAKTRSS), and the sequence was identical to the N-terminal sequence of human histone H2A (33). In addition, peak 1 and peak 2 comigrated with calf thymus histone H2B and H2A, respectively, on a tricine SDS-PAGE (data not shown). On the basis of the above results, we concluded that histones H2B (peak 1) and H2A (peak 2) are responsible for the major salt-resistant antimicrobial activity in the human placenta. This conclusion was further supported by immunoblot analyses, showing that peak 1 reacted strongly with the anti-histone H2B mAb and peak 2 reacted strongly with the anti-histone H2A mAb (data not shown).

Antimicrobial assays

The two isolated proteins were then tested for salt-resistant antimicrobial activity against *E. coli*. As shown in Table I, the MICs of purified histones H2A and H2B were determined in the presence of NaCl, and magainin 2 was included as a control for the comparison of salt-dependent antimicrobial activity. There was no significant increase in the MICs of histones H2A and H2B in the presence of up to 300 mM of NaCl, whereas magainin 2 showed a >10-fold increase in MIC under identical conditions.

LPS-binding and neutralization assays

The purified histones H2A and H2B from the human placenta were subjected to an LPS-binding assay, which showed that histone H2B bound to LPS five to six times stronger than did histone H2A (data not shown). This difference in the binding strength appears to result from different binding specificity of anti-histone H2A and anti-histone H2B mAbs (34) in the solid phase binding assay condition (see *Materials and Methods*). Therefore, the specificity of binding of histone H2B to the endotoxin LPS was examined. The LPS of Gram-negative bacteria is composed of three parts: the lipid A, a relatively conserved core oligosaccharide, and a terminal polysaccharide of variable length and composition that comprises

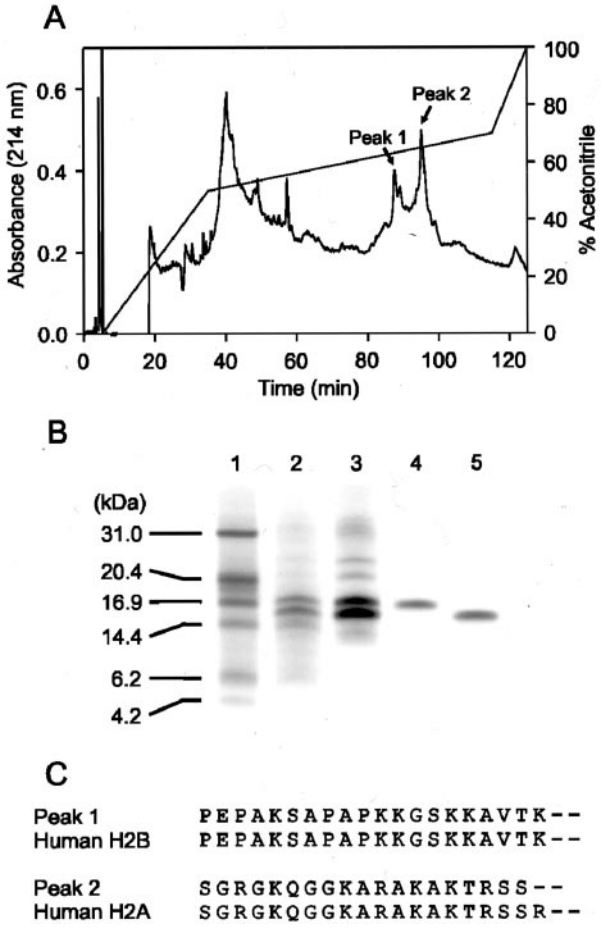

FIGURE 1. Purification of salt-resistant antimicrobial proteins. *A*, Reversed-phase HPLC chromatogram of the active fraction from a heparin-Sepharose column. The fraction with antimicrobial activity that was eluted with 1 M of NaCl from a heparin-Sepharose column was subjected to chromatography on a C4 reversed-phase HPLC column and fractionated with a 0–100% gradient of acetonitrile in 0.13% HFBA. Two active peaks, which are indicated by the arrows, were further separated by reversed-phase HPLC on the same column with slower gradient using 0.1% TFA as the ion-pairing agent. *B*, Tricine SDS-PAGE analysis of purified antimicrobial proteins. The purified proteins, named peak 1 and peak 2, were subjected to 10% tricine SDS-PAGE. *Lane 1*, m.w. marker; *lane 2*, crude extract from human placenta; *lane 3*, heparin-affinity chromatography fraction eluted with 1 M of NaCl; *lanes 4* and *5*, peak 1 and peak 2 purified by reversed-phase HPLC. *C*, Alignment of the N-terminal amino acid sequences of the purified antimicrobial proteins with those of H2A and H2B. The amino acid residues are shown using the single-letter codes. The amino acid sequences of peak 1 and peak 2 are aligned with those of human histone H2B and H2A, respectively.

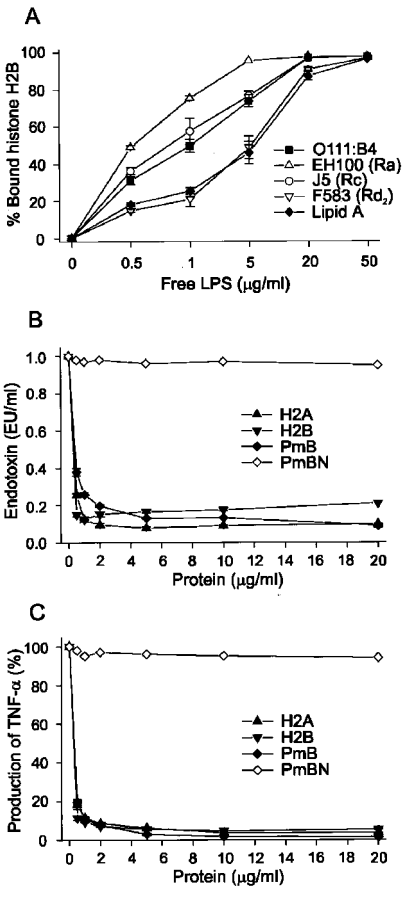

FIGURE 2. Characteristics of LPS binding and neutralization by histone proteins. *A*, Effect of various species of LPS on the binding of histone H2B to control LPS. The plate was coated with *E. coli* 0111:B4 LPS, and histone H2B (1 μg/ml) was added to the wells, along with the various LPS species. Binding is expressed as the percentage of histone H2B bound to the various types of LPS. A standard curve was generated for comparison by adding known amounts of histone H2B to the assay. *B*, Dose-dependent inhibition of endotoxin-induced LAL coagulation by histone proteins. LAL was incubated with 1 EU/ml *E. coli* 0111:B4 LPS in the presence or absence of the indicated amounts of histone proteins. A standard curve was generated by adding known amounts of endotoxin to the assay. *C*, Dose-dependent suppression by histone proteins of LPS-induced TNF-α secretion in RAW264.7 cells. RAW264.7 cells were incubated with 10 ng/ml *E. coli* 0111:B4 LPS in the presence or absence of the indicated amounts of histone proteins for 6 h. TNF-α in the supernatant was measured by ELISA. The LPS-stimulated value was used as 100%, and the data are represented as the percentage of inhibition of LPS-stimulated TNF-α production by the histone proteins. Each value represents the mean and SEM for the averages of duplicate samples from three independent experiments.

the *O*-specific Ag domain (35). To determine which part of the LPS is recognized by the purified histone H2B, inhibition assays were performed using LPS molecules with various polysaccharide chain lengths, ranging from lipid A alone (the smallest size), to Rd_2-LPS, Rc-LPS, and Ra-LPS, which consist of the lipid A moiety and variable parts of the core region (Rd_2-LPS < Rc-LPS < Ra-LPS), to 0111:B4 LPS, which has the complete lipid A, core, and *O*-Ag domains. As shown in Fig. 2*A*, the binding of histone H2B to LPS was inhibited progressively as the length of the core region of LPS decreased. The Ra-LPS, which lacks the *O*-Ag domains, bound more tightly than did 0111:B4 LPS. This finding indicates that the presence of the *O*-Ag domains either did not affect or was somewhat inhibitory to LPS binding. Lipid A showed an affinity to histone H2B similar to that of Rd_2-LPS. These results indicate that both the core region and the lipid A moiety of LPS are responsible for the LPS-binding activity of histone H2B.

To investigate the endotoxin-neutralizing activity of purified histones H2A and H2B, we compared their abilities to inhibit LPS-induced LAL coagulation and block LPS-induced TNF-α production by the RAW264.7 macrophage cell line. Inhibition of LPS-induced LAL coagulation by histones H2A and H2B is presented in Fig. 2*B*. The effects of PMB and PMBN on LPS-induced LAL coagulation are shown for comparison. We also tested the effects of other histones (data not shown). Histones H2A and H2B were more potent than the other histones tested (H1, H3, and H4) and PMB, on a molecular basis, in this endotoxin-neutralizing assay. The results of the TNF-α production assays are shown in Fig. 2*C*. RAW264.7 cells secrete TNF-α upon exposure to endotoxin (36).

A linear relationship between TNF-α secretion and endotoxin concentration was observed at LPS concentrations below 20 ng/ml, and a concentration of 10 ng/ml was selected for the following experiment. Mixing LPS with increasing concentrations of histones resulted in a dose-dependent suppression of LPS-induced TNF-α secretion (Fig. 2*C*). Similar to the results of the LAL assay, histones H2A and H2B exhibited a similar or slightly higher potency than did PMB in suppressing TNF-α secretion induced by LPS. Each reaction was performed in duplicate, and the reported values, expressed as percentage of inhibition of endotoxin activity, were calculated from the results of three assays executed on separate days.

As a control for any possible effects of trace amounts of impurities (e.g., organic compounds) on the antimicrobial and endotoxin-neutralizing activities of the purified histones H2A and H2B, we performed these assays (antimicrobial assay, LAL assay, and TNF-α production) with an irrelevant protein (m.w. of ~8000), purified by the same procedure, showing no antimicrobial activity. No effect was observed with an irrelevant protein under our experimental conditions. Furthermore, antimicrobial and endotoxin-neutralizing activities of calf thymus histones H2A and H2B were identical with those of the purified histones H2A and H2B. The effect of the purified proteins (an irrelevant protein, histones H2A and H2B) on the viability of RAW264.7 macrophages was also examined. The viability was not changed upon addition of the purified proteins (up to 25 μg/ml of final concentration) and at the end of the incubation (data not shown). Taken together, our results indicate that the effects of histones H2A and H2B on the antimicrobial and endotoxin-neutralizing activities are specific for the microbes and LPS-induced response.

Expression of histone proteins in human amnion epithelial cells and trophoblasts

To investigate the expression of histone proteins in human placenta in vivo, Formalin-fixed tissues were subjected to immunohistochemical analysis using anti-histone H2A and anti-histone H2B mAbs, and the results are shown for representative placental tissue sections (Fig. 3). The histones H2A and H2B proteins in second trimester placenta (20 wk of gestation) were prominent in the cytoplasm of syncytiotrophoblasts and amnion epithelial cells, where they were localized mainly on the surface epithelium. Chorionic cytotrophoblasts contained little or no H2A and H2B. Control isotype-matched IgG did not bind to any cells in placenta (data not shown). In term placenta, immunoreactivities with anti-histone H2A and anti-histone H2B mAbs were less intense in both syncytiotrophoblasts and amnion epithelial cells (data not shown). These results confirmed that expression of histones H2A and H2B occurs in the placenta during pregnancy, and indicate that the two proteins are expressed specifically at the maternal-fetal interface, with synthesis prominent in syncytiotrophoblasts and amnion epithelial cells.

Localization of histones H2A and H2B at the surface of amnion-derived WISH cells

The immunohistochemical analyses suggest that histones H2A and H2B are mainly localized at the surface epithelium of fetal membranes. To confirm this localization, we did immunocytochemistry using confocal immunofluorescence microscopy. Fig. 4 shows that both H2A and H2B are present at the surface epithelium of amnion-derived WISH cells. The mAbs stained nuclei of permeabilized WISH cells (Fig. 4, *A*, *C*, *G*, and *I*), as was expected, because histones H2A and H2B are nuclear Ags. In addition, immunoreaction of anti-histone H2A (Fig. 4, *A* and *C*) and anti-histone H2B mAbs (Fig. 4, *G* and *I*) with the cell surface was also observed. To

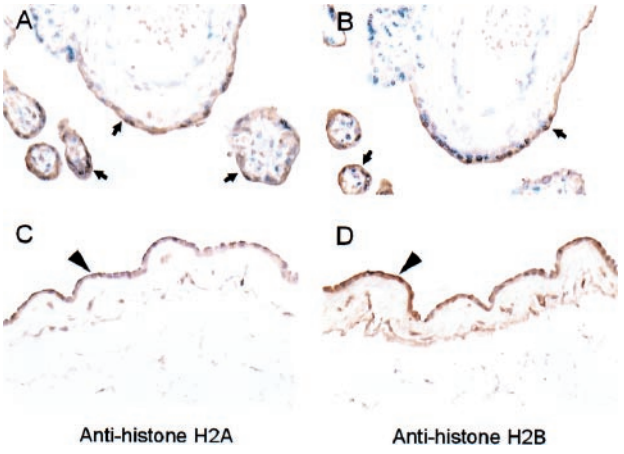

FIGURE 3. Immunohistochemical localization of histones H2A and H2B in human placenta. Sections of placental tissues were immunostained for histone H2A (*A* and *C*) and histone H2B (*B* and *D*). Histones H2A and H2B were localized in the cytoplasm of syncytiotrophoblasts (arrows; *A* and *B*) and amnion epithelial cells (arrowheads; *C* and *D*), where they were primarily associated with the surface epithelium. All sections are shown at a magnification of ×400. Similar results were noted in all placental tissues of varying gestational ages.

further verify the localization of histones H2A and H2B at the cell surface, the same experiment was performed with nonpermeabilized cells. Cells were fixed and immunolabeled without further Triton X-100 treatment. The anti-histone H2A (Fig. 4, *D* and *F*) and anti-histone H2B mAbs (Fig. 4, *J* and *L*) showed strong staining on the cell surface, clearly indicating the presence of cell surface-associated histones H2A and H2B in nonpermeabilized cells. Similar immunostaining results were also obtained in the LPS-treated WISH cells (data not shown). This observation supports the concept that histones H2A and H2B are synthesized and localized at the surface of amnion cells and released into the extracellular environment.

Extracellular release of histone H2B by amnion cells

We tested H2B production by amnion epithelial WISH cells. We obtained supernatants from cultured WISH cells treated with LPS and measured the accumulation of histone H2B in the culture supernatant over time with a sensitive ELISA. Fig. 5 shows that within 3 h of culture, there is a rapid appearance of histone H2B in the culture supernatants. Maximum histone H2B production was observed after ~24 h in culture in all LPS-treated culture samples. After 24 h, histone H2B concentrations in the supernatants ranged from 10 to 15 ng/ml, as determined by comparison with known amounts of a histone H2B standard. The levels of histone H2B production were not variable in response to LPS treatment, suggesting a constitutive production and secretion of histone H2B by WISH cells. We next studied whether antimicrobial concentrations of histones H2A and H2B could be generated under biologically relevant conditions. We measured the levels of histone H2B in human amniotic fluids by a specific ELISA. The concentrations of histone H2B from amniotic fluid were found to be 250 ± 67 ng/ml. The data are presented as the mean ± SEM for three separate experiments from 20 different normal individuals.

Histones H2A and H2B contribute to bactericidal activity of amniotic fluid

Amniotic fluid, like other body fluids, is known to possess inherent bactericidal properties (37, 38). When amniotic fluid (10 μl) was incubated with 1×10^3 CFU of *E. coli*, up to 100% of the exposed

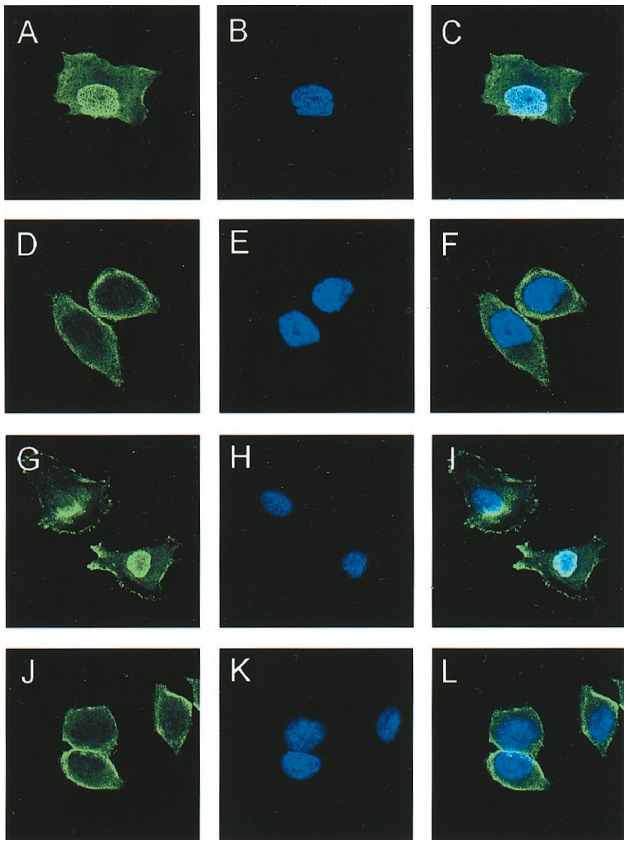

FIGURE 4. Immunocytochemical detection of histones H2A and H2B at the amnion WISH cell surface. Permeabilized cells (*A–C* and *G–I*) or nonpermeabilized cells (*D–F* and *J–L*) were treated with the anti-histone H2A (*A–F*) or anti-histone H2B mAbs (*G–L*), biotinylated secondary Ab, and streptavidin-FluorGreen conjugate in sequence (green, *left panel* of each row). The nuclei were counterstained with DAPI (blue, *middle panel* of each row). Merged images were generated by computer-assisted superimposing FluorGreen and DAPI images (*right panel* of each row). The confocal laser photomicrographs show the distribution of histones H2A and H2B at the surface epithelium as well as within the nuclei of WISH cells. The magnification is ×400. The data shown are representative of three independent experiments. WISH cells incubated with the mouse isotype-matched IgG, as a negative control, showed no reactivity (data not shown).

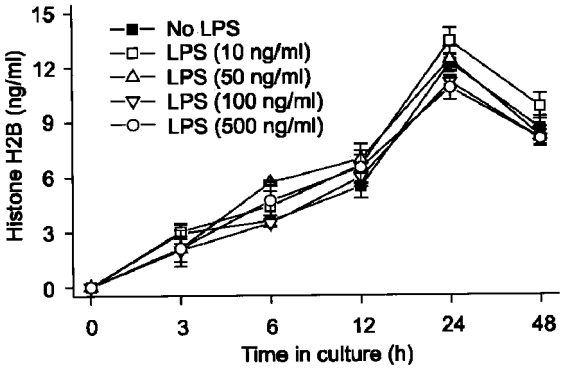

FIGURE 5. Secretion of histone H2B in supernatants from cultured WISH cells. Amnion-derived WISH cells were cultured at 10^5 cells/ml for 48 h, and the concentration of histone H2B in the cultured supernatants was quantitated with a highly specific ELISA. The secretion of histone H2B in the supernatant reached a maximum at 24 h and did not change in response to LPS treatment. The observed H2B concentrations were expressed as the mean ± SEM for duplicate samples from three independent experiments.

microorganisms were killed (Fig. 6*A*), and a similar result was obtained when amniotic fluid was incubated with group B *Streptococcus* (Fig. 6*B*). To address the contribution of histones H2A and H2B to the bactericidal activity of amniotic fluid, we used specific Abs to block the antimicrobial activity of histones H2A and H2B in amniotic fluid. The mAbs to histones H2A and H2B neutralized up to 65% and 58% of the bactericidal activity of amniotic fluid incubated with *E. coli* or group B *Streptococcus*, respectively (Fig. 6). The bactericidal activity of amniotic fluid was inhibited dose dependently by the mAbs to H2A and H2B. Consistent with these findings, the bactericidal activity of amniotic fluid was almost unaffected by control isotype-matched IgG. From these experimental results, we could infer that amniotic fluid maintains amniotic sterility during bacterial infection, and histones H2A and H2B contribute in part to the bactericidal activity of amniotic fluid.

Discussion

In this study, we have demonstrated that the salt-resistant antimicrobial and endotoxin-neutralizing proteins of the human placenta are histones H2A and H2B. These proteins may play an important role in protection of the fetus from invading microorganisms. Histones H2A and H2B were bactericidal for our *E. coli* test strain even in the presence of high salt (up to 300 mM of NaCl). NaCl is the most predominant salt in vivo, and the ability to resist salt is important for host defense proteins to function under physiological

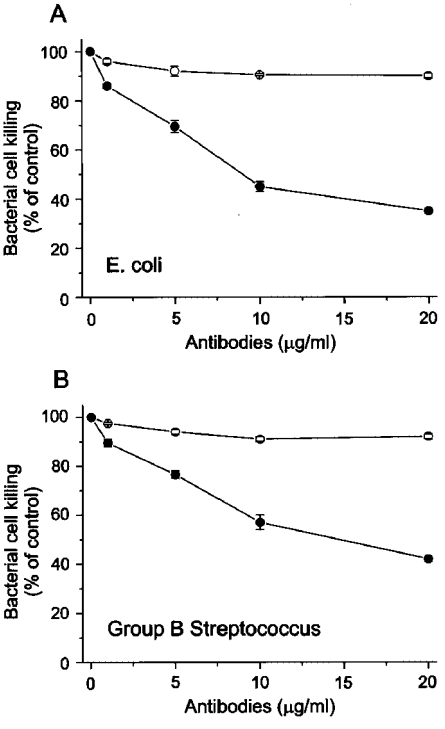

FIGURE 6. Contribution of histones H2A and H2B to bactericidal activity of amniotic fluid. Amniotic fluid (10 μl) was combined in triplicate with mAbs to histones H2A and H2B or with control isotype-matched IgG (10–200 μg/ml) for 30 min and analyzed for bactericidal activity against 1×10^3 CFU of *E. coli* (*A*) or group B *Streptococcus* (*B*). After 30 min at 37°C, surviving CFU were quantified by growth on semisolid media at 37°C overnight. The bactericidal activity of amniotic fluid was inhibited dose dependently by the mAbs to H2A and H2B. Bactericidal activity is expressed as percentage of bacteria killed relative to bacteria unexposed to amniotic fluid ± SEM. ●, Amniotic fluid with mAbs to H2A and H2B; ○, amniotic fluid with control isotype-matched IgG.

conditions. In the case of cystic fibrosis, it has been suggested that the susceptibility of epithelial antimicrobial proteins (presumably β-defensins) to inhibition by salt explains the persistence of chronic *Pseudomonas aeruginosa* infections in the lungs of patients with this disease (39). Mounting evidence indicates that antimicrobial proteins play key roles in the innate immune defenses of vertebrates. However, their activity is often abrogated under physiological conditions (100–150 mM of NaCl) (20, 40). A sodium concentration of 135 mM has been reported to be present in the amniotic fluid of human placenta (41). Therefore, the findings of this study support the hypothesis that retention of antimicrobial activity in physiological salt concentrations is important for host defense in the placental physiological environment.

In addition to their antimicrobial activity, naturally occurring cationic proteins have been shown to bind LPS and block its ability to stimulate the production of TNF-α, IL-6, and other inflammatory mediators (42–44). Systemic disease associated with the presence of pathogenic microorganisms or their toxins in the blood often involves Gram-negative bacteria and the release of a bacterial outer membrane component, an endotoxin (45). The toxicity of the endotoxin, which is also known as LPS, is contained within the lipid A domain (46). The physiological mechanism whereby endotoxin exerts its effect on humans involves the release of cytokines, most significantly TNF-α (47). In the human placenta, LPS is a potent biological product capable of inducing PG release from several cell types and, therefore, may be involved in the onset of human parturition when an intra-amniotic infection exists (48–50). Given that a heparin-binding lectin from human placenta was hypothesized to be histone H2B (51) and that a neuronal LPS-binding protein was identified as histone H1 (52), we were prompted to investigate the LPS binding and neutralization abilities of histones H2A and H2B. To date, the majority of studies on host defense involving histone proteins have been focused on broad-spectrum antimicrobial activities (24, 53, 54). The interaction of histone proteins with LPS and the influence of the *O*-Ag, core, or lipid A domains of LPS on LPS binding to histones had heretofore not been evaluated. To characterize the binding of histone proteins to LPS, we used a solid phase binding assay with microtiter plates whose binding surfaces were coated with bacterial LPS. Using this assay, we found that histone H2B binds avidly to many different LPS isolates from smooth and rough strains of *E. coli*, with a high degree of specificity for the core and lipid A domains of bacterial LPS.

However, the binding ability alone appears to be a deceptive indicator of endotoxin-neutralizing activity, as molecules with nearly the same binding propensities could either opsonize or sequester the endotoxin (55, 56). This is clearly evident in the comparison of the antiendotoxic activity of PMB and its derivative PMBN (57). The only difference between PMB and PMBN is the absence of the 6-heptanoyl/octanoyl diaminobutyryl group at the amino terminus of the latter. This difference is responsible for the poor antiendotoxic activity of PMBN (58), despite apparent similarities in LPS binding ability (59). Therefore, we examined directly the antiendotoxic activity of histones H2A and H2B. Histones H2A and H2B showed dose-dependent inhibition of the endotoxin activity of LPS comparable with that of PMB. Histones H2A and H2B most likely exert their antiendotoxin actions by binding to the lipid A moiety and, consequently, blocking the biological effects of endotoxin. It is known that the lipid A portion of endotoxin is responsible for the activation of LAL (60) and stimulation of TNF-α secretion (46). This notion is further supported by our observation that histone H2B bound directly to lipid A. Given the fact that most known antiendotoxin proteins are cationic in nature and that both the lipid A and oligosaccharide core portions of LPS are anionic, one may postulate that electrostatic forces contribute to the binding of LPS to the neutralizing cationic histone proteins. Because the *E. coli* lipid A domain investigated in this study is similar to that of many Gram-negative pathogens, it is probable that histones H2A and H2B would also bind to and neutralize the lipid A region of many of the clinical isolates of Gram-negative pathogens taken from human placental tissues. By analogy with LPS-binding antimicrobial peptides (61, 62), histone H2A or H2B binding to or complex formation with free LPS released from dividing or dying Gram-negative bacteria could also block the LPS-induced synthesis and secretion of cytokines such as TNF-α that frequently lead to fatal septic shock.

Because the histone H2A and H2B proteins are normally expressed predominantly in eukaryotic nuclei, we performed further studies to localize the presence of histones H2A and H2B in placental tissues. Immunohistochemistry on tissue sections showed that in addition to being present in nuclei, histones H2A and H2B were also present in the cytoplasm of syncytiotrophoblasts and amnion epithelial cells. Of note is the finding that histones H2A and H2B were localized on the extracellular surface, forming band-like barriers on the amnion and trophoblast layers. The trophoblast layer, which forms the outer layer of chorion, comprises both cytotrophoblasts and syncytiotrophoblasts, and the syncytiotrophoblasts represent differentiated, fused cytotrophoblasts (63). Syncytiotrophoblasts, which are continuously bathed in maternal blood, were one of the major cell types that contained immunoreactive histones H2A and H2B. Thus, histones H2A and H2B are likely to play important roles in maintaining the barrier function of the immune-privileged placenta. The coating of histones H2A and H2B on the syncytiotrophoblast layer may serve to limit the infection and colonization of microbial pathogens via the hematogenous route. The chorion and amnion epithelial cells form the outer and inner layers of the chorioamnion, respectively. These layers are separated by a collagen-rich stroma and by basement membranes. The chorion and amnion cells form two important cellular barriers protecting the fetus from bacterial infection. Recent studies suggest that the amnion epithelial cell monolayer may provide an effective barrier against entry of microbial pathogens to the nutrient-rich amniotic fluid (64), and may partially explain why the rate of amniotic fluid infections associated with chorioamnionitis is not higher (65, 66). Moreover, bacterial LPS fails to cross the chorioamniotic membranes, possibly due to the presence of LPS-binding proteins (67). We speculate, therefore, that the failure of both microbial pathogens and bacterial LPS to cross the placental membranes may be attributed in part to the antimicrobial and endotoxin-neutralizing potential of histone H2A and H2B proteins coating the epithelial surface of the placenta.

Recently, the presence of histones in both the cytoplasm and nucleus has been reported (24, 53, 54, 68, 69), and the secretion of histone proteins into the extracellular milieu was also observed (24, 70–72). It is becoming clear that histone proteins are present in both the cytoplasm and the nucleus of rapidly regenerating or transcriptionally active cells such as placental, gastrointestinal (24, 54), and skin (73, 74) epithelial cells. Indeed, we detected nuclear staining of some syncytiotrophoblasts and amnion epithelial cells with anti-phospho-histone H3 Ab, a mitosis marker (data not shown). Histone H3 phosphorylation at Ser^{10} correlates temporally with mitosis in mammalian cells (75, 76).

It appears that the expression of histones H2A and H2B is not up-regulated during infections in human placenta. In fact, our findings indicate that histones H2A and H2B are constitutively produced in cultured WISH cells. The constitutive presence of histones H2A and H2B at fetal membrane is most likely relevant for immediate defense response, since de novo synthesis of histones

H2A and H2B is not needed when bacterial intruders enter the placenta. The antimicrobial and endotoxin-neutralizing potentials of histones H2A and H2B may contribute to the establishment of a local defense barrier at fetal membrane against microbial infection and LPS-induced inflammation, causing preterm birth and neonatal fetal demise. It has been reported that improved host defense is likely to have favored the constitutive expression of antimicrobial proteins in tissues that are continually exposed to infections or that are especially important, e.g., those of the reproductive tract (28, 77, 78). Goldman et al. (39) reported that antimicrobial activity in airway surface fluid of human bronchial xenografts is almost diminished by antisense inhibition of constitutively expressed human β-defensin-1. The mean value for the concentration of histone H2B in amniotic fluid is ~250 ng/ml. Considering that the amount of histone H2A is similar to that of histone H2B in the placental tissues (Fig. 1*B*), the total concentration of histones H2A and H2B in amniotic fluid may be ~500 ng/ml, which is much lower than MIC value of 10 μg/ml. However, the MIC value under our experiments was determined with an inoculum size of 5×10^4 CFU of *E. coli*. Studies on measuring the antimicrobial potency of histones H2A and H2B by CFU counting assay showed that the total concentration (500 ng/ml) of histones H2A and H2B was enough to inhibit the growth of an inoculum size of 5–10 $\times$ 10^2 CFU of *E. coli* (data not shown). From this observation, we speculate that the concentration of histones H2A and H2B in amniotic fluid may be sufficient to be microbicidal for a small number of microorganisms that breached constitutive defenses at amniotic membranes and gained access to the intra-amniotic cavity. The histones H2A and H2B may be concentrated at the sites of their secretion onto the negatively charged epithelial surface, and the actual histone concentrations at the site of placental infection might be substantially higher. Further studies are necessary to evaluate the functional contributions of histones H2A and H2B to innate immune defenses in human placenta in vivo.

In conclusion, the antimicrobial and endotoxin-neutralizing activities of histones H2A and H2B and their detection in syncytiotrophoblasts and amnion cells suggest the existence of a novel mechanism by which the fetus and placenta are protected against microbial infection. Therefore, histones H2A and H2B may form an important and previously unrecognized defense for the immune-privileged human placenta.

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